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Please type a plus sign (+) inside this box → ☐**UTILITY  
PATENT APPLICATION  
TRANSMITTAL**

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No. 240042052403

Total Pages -62-

First Named Inventor or Application Identifier

Jeffrey S. GLENN

Express Mail Label No. EL628462123US

**CERTIFICATE OF MAILING BY "EXPRESS MAIL"**

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Silvia E. Pearce**APPLICATION ELEMENTS**

See MPEP chapter 600 concerning utility patent application contents.

ADDRESS TO:

Assistant Commissioner for Patents  
Box Patent Application  
Washington, DC 20231

1. ☒ Fee Transmittal Form  
(Submit an original, and a duplicate for fee processing)
2. ☒ Specification [Total Pages  ]  
(preferred arrangement set forth below)
- Descriptive title of the invention
  - Cross References to Related Applications
  - Statement Regarding Fed sponsored R & D
  - Reference to Microfiche Appendix
  - Background of the Invention
  - Brief Summary of the Invention
  - Brief Description of the Drawings (if filed)
  - Detailed Description
  - Claim(s)
  - Abstract of the Disclosure
3. ☒ Drawing(s) (35 USC 113) [Total Sheets  ]
4. ☒ Oath or Declaration [Total Pages  ]
- a. ☐ Newly executed (original or copy)
  - b. ☒ Copy from a prior application (37 CFR 1.63(d)  
(for continuation/divisional with Box 17 completed)  
[Note Box 5 below]
  - i. ☐ DELETION OF INVENTOR(S)  
Signed statement attached deleting inventor(s) named in  
the prior application, see 37 CFR 1.63(d)(2) and 1.33(b)
5. ☒ Incorporation By Reference (useable if Box 4b is checked)  
The entire disclosure of the prior application, from which a copy of the  
oath or declaration is supplied under Box 4b, is considered as being  
part of the disclosure of the accompanying application and is hereby  
incorporated by reference therein

6. ☐ Microfiche Computer Program (Appendix)
7. ☒ Nucleotide and/or Amino Acid Sequence Submission  
(if applicable, all necessary)
- a. ☒ Computer Readable Copy
  - b. ☒ Paper Copy (identical to computer copy)
  - c. ☒ Statement verifying identity of above copies

**ACCOMPANYING APPLICATION PARTS**

8. ☐ Assignment Papers (cover sheet & document(s))
9. ☐ 37 CFR 3.73(b) Statement ☐ Power of Attorney  
(when there is an assignee)
10. ☐ English Translation Document (if applicable)
11. ☒ Information Disclosure  
Statement (IDS)/PTO-1449 ☐ Copies of IDS  
Citations
12. ☐ Preliminary Amendment
13. ☒ Return Receipt Postcard (MPEP 503)  
(Should be specifically itemized)
14. ☐ Small Entity ☒ Statement filed in prior application,  
Statement(s) Status still proper and desired
15. ☐ Certified Copy of Priority  
Document(s) (if foreign priority is  
claimed)
16. ☐

17. If a CONTINUING APPLICATION, check appropriate box and supply the requisite information:

☐ Continuation ☒ Divisional ☐ Continuation-in-part (CIP) of prior application No: 09/028,655**18. CORRESPONDENCE ADDRESS**

Peng Chen  
Registration No. (43,543)  
Morrison & Foerster LLP  
12636 High Bluff Drive  
Suite 300  
San Diego, California 92130-2071  
Telephone: (858) 720-5117  
Facsimile: (858) 720-5125

- ☒ If a paper is untimely filed in the above-referenced application by applicant or his/her representative, the Assistant Commissioner is hereby petitioned under 37 C.F.R. § 1.136(a) for the minimum extension of time required to make said paper timely. In the event a petition for extension of time is made under the provisions of this paragraph, the Assistant Commissioner is hereby requested to charge any fee required under 37 C.F.R. § 1.17(a)-(d) to **Deposit Account No. 03-1952**. However, the Assistant Commissioner is **NOT** authorized to charge the cost of the issue fee to the Deposit Account.

The filing fee has been calculated as follows:

FOR	NUMBER FILED	NUMBER EXTRA	RATE	CALCULATIONS
TOTAL CLAIMS	9 - 20 =	0	x \$18.00	\$0
INDEPENDENT CLAIMS	1- 3 =	0	x \$78.00	\$0
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$260.00	\$0
			BASIC FEE	\$710.00
			TOTAL OF ABOVE CALCULATIONS =	\$710.00
Reduction by 1/2 for filing by small entity (Note 37 C.F.R. §§ 1.9, 1.27, 1.28). If applicable, verified statement must be attached.				<b>\$355.00</b>
Assignment Recording Fee (if enclosed)				\$0
			TOTAL =	<b>\$355.00</b>

- ☒ A check in the amount of **\$355.00** is attached.
- ☐ Charge \$355.00 to **Deposit Account No. 03-1952** referencing docket no. 240042052403.
- Applicant(s) hereby petitions for any required relief including extensions of time and authorizes the Assistant Commissioner to charge the cost of such petitions and/or other fees or to credit any overpayment to **Deposit Account No. 03-1952** referencing docket no. 240042052403. A duplicate copy of this transmittal is enclosed, for that purpose.

Dated: October 13, 2000

Respectfully submitted,

By: \_\_\_\_\_

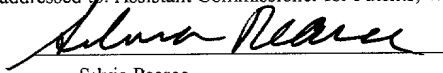
Peng Chen  
Registration No. (43,543)  
Morrison & Foerster LLP  
12636 High Bluff Drive  
Suite 300  
San Diego, California 92130-2071  
Telephone: (858) 720-5117  
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Silvia Pearce

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In the application of:

Jeffrey S. Glenn

Serial No.: To be assigned

Divisional of Serial No. 09/028,655

Filing Date: Herewith

For: METHOD FOR INHIBITION OF VIRAL  
MORPHOGENESIS

Examiner: To be assigned

Group Art Unit: To be assigned

**PRELIMINARY AMENDMENT**

Assistant Commissioner for Patents  
Washington, D.C. 20231

Dear Sir:

Preliminary to the examination of the above-captioned application, please amend the application as follows. Also enclosed are the following exhibits:

Exhibit A: Continental Can Co. USA, v. Monsanto Co., 948 F.2d 1264; 20 U.S.P.Q.2d 1746 (Fed. Cir. 1991);

Exhibit B: In re Oelrich, 666 F.2d 578, 212 U.S.P.Q. 322 (CCPA 1981); and

Exhibit C: Hansgrig v. Kemmer, 102 F.2d 212, 40 U.S.P.Q. 665 (CCPA 1939).

**IN THE SPECIFICATION:**

Please insert, on page 1, after the title:

--This application is a divisional of U.S. Serial No. 09/028,655, filed February 24, 1999, now allowed, which is a continuation of U.S. Serial No. 08/347,448, filed June 23, 1995, now U.S. Patent No. 5,876,920, which is a 371 of PCT/US93/05247, filed June 1, 1993, which is a Continuation-in-Part of U.S. Serial No. 07/890,754, filed May 29, 1992, now U.S. Patent No. 5,503,973.--

- On page 1, line 27, after "CXXX" insert --(SEQ ID NO:1)--;
- On page 2, line 7, after "CXXX" insert --(SEQ ID NO:1)--;
- On page 2, line 10, after "CXXX" insert --(SEQ ID NO:1)--;
- On page 2, line 19, after "CXXX" insert --(SEQ ID NO:1)--;
- On page 2, line 20, after "Cys-Arg-Pro-Gln" insert --(SEQ ID NO:2)--;
- On page 2, line 21, after "CXXX" insert --(SEQ ID NO:1)--;
- On page 2, line 22, after "CXXX" insert --(SEQ ID NO:1)--;
- On page 5, line 4, after "Cys-Arg-Pro-Gln-COOH" insert --(SEQ ID NO:2)--;
- On page 5, line 5, after "CXXX" insert --(SEQ ID NO:1)--;
- On page 5, line 17, after "CXXX" insert --(SEQ ID NO:1)--;
- On page 7, line 7, after "CXXX" insert --(SEQ ID NO:1)--;
- On page 7, line 15, after "CXXX" insert --(SEQ ID NO:1)--;
- On page 7, line 20, after "Cys-Arg-Pro-Gln-COOH" insert --(SEQ ID NO:2)--;
- On page 7, line 21, after "CXXX" insert --(SEQ ID NO:1)--;
- On page 9, line 1, after "CXXX" insert --(SEQ ID NO:1)--;
- On page 9, line 15, after "CXXX" insert --(SEQ ID NO:1)--;
- On page 9, line 16, after "CXXX" insert --(SEQ ID NO:1)--;
- On page 10, line 1, after "(CRPQ)" insert --(SEQ ID NO:2)--;
- On page 10, line 10, after "CXXX" insert --(SEQ ID NO:1)--;

On page 10, line 30, after "CXXX" insert --(SEQ ID NO:1)--;

On page 10, line 34, after "CXXX" insert --(SEQ ID NO:1)--;

On page 11, line 30, after "C-A-A-X" insert --(SEQ ID NO:3)--;

On page 11, line 33, after "CXXX" insert --(SEQ ID NO:1)--;

On page 12, line 2, after "XCXX, XXCX, and XXXC" insert --(SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6)--;

On page 13, line 19, after "CXXX" insert --(SEQ ID NO:1)--;

On page 13, line 21, after "CXXX" insert --(SEQ ID NO:1)--;

On page 13, line 23, after "CXXX" insert --(SEQ ID NO:1)--; and

On page 14, line 17, after "CXXX" insert --(SEQ ID NO:1)--.

**IN THE CLAIMS:**

Please cancel claims 1-12.

Please add the following new claims 13-21:

13. (New) A method to treat a viral infection in a subject via inhibiting the prenylation of a protein contained in the virus infecting said subject, which method comprises administering to said subject an agent selected from the group consisting of
- a peptide that mimics the amino acid sequence of a "CXXX" (SEQ ID NO:1), "XCXX" (SEQ ID NO:4), "XXCX" (SEQ ID NO:5), or "XXXC" (SEQ ID NO: 6) box as it occurs in said viral protein,
  - an inhibitor of enzymes included in the pathway of the prenyl lipid synthesis from mevalonate,
  - an inhibitor of a prenyl transferase, and
  - a mimic of a prenyl group.

14. (New) The method of claim 13, wherein said agent mimics the amino acid sequence of the "CXXX" (SEQ ID NO:1), "XCXX" (SEQ ID NO:4), "XXCX" (SEQ ID NO:5), or "XXXC" (SEQ ID NO: 6) box as it occurs in said protein.

15. (New) The method of claim 13, wherein said agent is an inhibitor of enzymes along the pathway of prenyl lipid synthesis from mevalonate.

16. (New) The method of claim 13, wherein said agent is an inhibitor of a prenyltransferase.

17. (New) The method of claim 13, wherein said agent is a mimic of a prenyl group.

18. (New) The method of claim 13, wherein said subject is an animal or a plant.

19. (New) The method of claim 18, wherein said animal is a mammal.

20. (New) The method of claim 19, wherein said mammal is a human.

21. (New) The method of claim 19, wherein said mammal is a non-human primate.

**IN THE ABSTRACT:**

On page 20, line 11, after "CXXX" insert --(SEQ ID NO:1)--.

**REMARKS**

Entry of the attached Sequence Listing (pages 1-3) is respectfully requested. The paper copy of the Sequence Listing is identical to the Sequence Listing submitted in computer readable form. No new matter is added.

Upon entry of the present Preliminary Amendment, claims 13-21 will be pending. Claims 1-12 are canceled without any prejudice and disclaimer. Applicant reserves his rights to pursue the subject matter of the cancelled claims in a subsequent application. Claims 13-21 are

directed to the Group IV of the non-elected claims in the parent application, U.S. Serial No. 09/028,655, filed February 24, 1999. Support for reciting “CXXX”, “XCXX”, “XXCX”, or “XXXC” box in claim 13 can be found, *inter alia*, at page 11, line 30 through page 12, line 6 of the present specification. Support for reciting “an animal or a plant”, “a mammal”, “a human”, or “a non-human primate” in claims 18-21 can be found, *inter alia*, at page 3, lines 14-14 and at page 15, lines 3-5 of the present specification. Therefore, the above-described amendments do not introduce any new matter into the present application.

**Rightsel, even in view of Van der Pyl, does not inherently disclose, teach or suggest that the “anti-viral” activity and the farnesylation inhibition activity of gliotoxin and acetylgliotoxin are the same**

In the prosecution of the parent application, Rightsel et al., Nature (1964) 204:1333-1334 (“Rightsel”) was cited as a prior art. It is submitted herein that Rightsel does not inherently disclose, teach or suggest the presently pending claims for several reasons. First, Rightsel provides no true measure of actual virus production, titers, genome levels, etc. The “anti-viral” activity measured in Rightsel is simply a visual assessment of cell appearance and is nothing more than a host cell response to the presence of viral proteins. The cytopathic effect described in Rightsel can best be viewed as a morphologic correlation to early cytotoxicity of the treatment with gliotoxin and acetylgliotoxin. This cytotoxicity could be inhibited by numerous conditions, *e.g.*, pH changes, protein synthesis inhibition, etc., none of which would have any relevance to the presently claimed methods.

In addition, even assuming, *arguendo*, that Rightsel discloses, teaches or suggests the kind of anti-viral activity contemplated by the presently pending methods, Rightsel still does not disclose, teach or suggest, at least explicitly, that gliotoxin and acetylgliotoxin have any farnesylation inhibiting activity. Van der Pyl et al., J. Antibiotics (1992) 45:1802-1805 (“Van der Pyl”), which is not a prior art to the present application, was used as an extrinsic evidence to show that Rightsel inherently discloses, teaches or suggests that gliotoxin and acetylgliotoxin have farnesylation inhibiting activity and this farnesylation inhibiting activity is the basis for the arguably disclosed or taught “anti-viral activity” of gliotoxin and acetylgliotoxin. However, such a conclusion cannot be reached in view of the total disclosure of Rightsel and Van der Pyl pursuant to the legal precedents on the “inherency” issue.

The Court in Continental Can Co. USA, v. Monsanto Co., 948 F.2d 1264; 20 U.S.P.Q.2d 1746 (Fed. Cir. 1991) (Exhibit A) stated:

To serve as an anticipation when the reference is silent about the asserted inherent characteristic, such gap in the reference may be filled with recourse to extrinsic evidence. Such evidence must make clear that the missing descriptive matter is necessarily present in the thing described in the reference, and that it would be so recognized by persons of ordinary skill. *In re Oelrich*, 666 F.2d 578, 581, 212 U.S.P.Q. 322, 326 (CCPA 1981) (Exhibit B) (quoting *Hansgrig v. Kemmer*, 102 F.2d 212, 214, 40 U.S.P.Q. 665, 667 (CCPA 1939) (Exhibit C) provides:

Inherency, however, may not be established by probabilities or possibilities. The mere fact that a certain thing *may* result from a given set of circumstances is not sufficient. [Citations omitted.] If, however, the disclosure is sufficient to show that the natural result flowing from the operation as taught would result in the performance of the questioned function, it seems to be well settled that the disclosure should be regarded as sufficient.

Rightsel discloses or teaches that gliotoxin and acetylgliotoxin have certain “activity” against, besides HSV and influenza viruses, poliovirus, which is not a prenylated virus. This result indicates that whatever this activity is, it has nothing to do with farnesylation inhibiting activity. In addition, as discussed in the Amendment under 37 C.F.R. § 1.116, filed April 7, 2000 in connection with the parent application, Van der Pyl, if not conclusively proves, strongly indicates that the “anti-viral” activity and farnesylation inhibiting activity of gliotoxin and acetylgliotoxin are two separate and distinct activities. Further, Van der Pyl actually indicates that the “anti-viral activity” and the farnesylation inhibiting activity of gliotoxin and acetylgliotoxin are two mutually exclusive activities, *i.e.*, the oxidized forms have the “anti-viral” activity while the reduced forms have the farnesylation inhibiting activity. Taken as a whole, it cannot be said that Rightsel, even in view of Van der Pyl, necessarily discloses or teaches to the skilled artisans that the “anti-viral” activity and farnesylation inhibiting activity of gliotoxin and acetylgliotoxin are the same one because there exists plenty of contrary evidence. Rightsel, in view of Van der Pyl, may possibly or even probably discloses or teaches that such is the case, but as the CCPA and Federal Circuit consistently held, mere “probabilities or possibilities” is not sufficient to establish “inherency” by extrinsic evidence.



## CONCLUSION

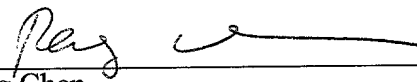
In view of the above remarks, it is respectfully submitted that the pending claims 13-21 are in form of allowance. Early allowance of the pending claims 13-21 are earnestly requested.

In the unlikely event that the transmittal letter is separated from this document and the Patent Office determines that an extension and/or other relief is required, applicant petitions for any required relief including extensions of time and authorizes the Assistant Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to **Deposit Account No. 03-1952** referencing docket no. 240042052403. However, the Assistant Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Respectfully submitted,

Dated: October 13, 2000

By:

  
Peng Chen  
Registration No. 43,543

Morrison & Foerster LLP  
12636 High Bluff Drive  
Suite 300  
San Diego, California 92130-2071  
Telephone: (858) 720-5117  
Facsimile: (858) 720-5125

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5            METHOD FOR INHIBITION OF VIRAL MORPHOGENESIS

          This invention was made with the support of the  
National Institutes of Health. The United States  
Government has certain rights in this invention.

10

Technical Field

          The invention is directed to inhibiting viral  
morphogenesis and viral infection. In particular, it  
concerns effecting such inhibition by inhibiting the  
15    prenylation or post prenylation reactions of a viral  
protein.

Background Art

          It has been shown that certain membrane-  
20    associated proteins require the addition of lipophilic  
residues in order to function properly. One family of  
such modifications is termed "prenylation" because the  
hydrophobic residue is derived from isoprenoid  
precursors. The prenyl residue is known to attach to the  
25    sulfhydryl group of a cysteine which has been shown in a  
number of membrane-associated proteins to be contained in  
a "CXXX" box at the carboxy terminus of the substrate  
protein. In particular, one such membrane-associated  
30    protein has been shown to be the protein product of the  
ras oncogene. Summaries of these reactions conferring  
hydrophobic properties on membrane proteins, including  
prenylation, have appeared by Hoffman, M., Science (1991)  
254:650-651, and by Gibbs, J.B., Cell (1991) 65:1-4.

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In addition, in many cases, prenylation is a first step in a series of further reactions which modify the carboxy terminus of prenylated proteins. These prenylation initiated, or post-prenylation reactions  
5 include carboxymethylation and proteolysis.

In the prenylation substrate proteins studied to date, the CXXX box contains aliphatic residues in the second and third positions and a leucine, serine, methionine, cysteine or alanine in the terminal position.  
10 Thus, in the CXXX boxes so far studied, the box itself is relatively hydrophobic.

It has now been found that prenylation of a viral protein is necessary for the morphogenesis of hepatitis delta virus (HDV). This is the first  
15 demonstration that viral proteins are subject to prenylation. Furthermore, certain functional consequences can be ascribed to prenylation. The viral protein which is the target of prenylation, surprisingly, contains a hydrophilic CXXX box of the sequence  
20 Cys-Arg-Pro-Gln. Prenylation, or prenylation-initiated modification, of this relatively hydrophilic CXXX box and corresponding CXXX boxes (hydrophilic or otherwise) or other cysteine-containing sequences near the C-terminus of proteins in other virions are suitable targets for  
25 antiviral strategies.

These targets can now be seen to include, but are not limited to, proteins of hepatitis A virus (HAV), hepatitis C virus (HCV), herpes simplex virus (HSV), cytomegalovirus (CMV), varicella-zoster virus (VZV),  
30 influenza virus, plant viruses such as tobacco mosaic satellite virus (TMSV) and barley stripe mosaic virus (BSMV), the core antigen of hepatitis B virus (HBV) and the nef gene product of human immunodeficiency virus-1 (HIV-1) -- especially since nef has been shown to play an  
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important role in the development of AIDS. (Kesstler, H.W. III, et al. Cell (1991) 65:651-662. Accordingly, inhibition of the prenylation of these target proteins or the post-prenylation reactions thereof is claimed to be  
5 inhibitory to the progress of these infections.

#### Disclosure of the Invention

The invention provides methods to interfere with viral morphogenesis, production, release or  
10 uncoating both *in vitro* and *in vivo*. Agents which interfere with the prenylation of, or the post-prenylation reactions of, at least one viral protein are provided to infected cells to halt the viral infection. Such cells may be in culture or may be contained in an  
15 animal or plant subject.

Thus, in one aspect, the invention is directed to a method to inhibit viral morphogenesis, production, release or uncoating which method comprises effectively interfering with the prenylation of, or the post-prenylation reactions of, at least one viral protein. In  
20 another aspect, the invention is directed to an assay method for screening candidate drugs for their ability to inhibit prenylation. In a third aspect, the invention is directed to a method for treating viral infection by  
25 administering an agent effective to inhibit prenylation of, or the post-prenylation reactions of, a viral protein. In preferred embodiments, the viral protein is the large delta antigen of the hepatitis D virus, core antigen of HBV, or the nef protein of HIV.

#### Brief Description of the Drawings

Figures 1A and 1B are photocopies of immunoblots of proteins obtained by lysis of viral-  
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infected cells expressing viral proteins and treated with tritiated mevalonate.

Figures 2A and 2B are photocopies of immunoblots of proteins derived from lysates of cells  
5 containing wild type or mutant viral proteins and labeled with tritiated proline or mevalonate.

Figures 3A, 3B, 3C and 3D are photocopies of immunoblots of various cell supernatants containing viral proteins.

10 Figure 4 is a diagrammatic representation of the progress of HDV morphogenesis.

#### Modes of Carrying Out the Invention

Hepatitis delta virus (HDV) infections cause  
15 both acute and chronic liver disease and can be fatal (1, 2). This RNA virus contains a 1.7 kb single-stranded circular genome and delta antigen, the only known HDV-encoded protein. These elements are encapsulated by a lipid envelope in which hepatitis B virus surface  
20 antigens are embedded (3), which explains why HDV infections occur only in the presence of an accompanying HBV infection (4, 5). Two isoforms of delta antigen exist in infected livers and serum (6, 7). This heterogeneity arises from a unidirectional mutation at a  
25 single nucleotide in the termination codon for delta antigen (codon 196: UAG → UGG), which occurs during replication (8). Thus, although small delta antigen is 195 amino acids long, large delta antigen is identical in  
30 acids at its COOH terminus. Although both forms of delta antigen contain the same RNA genome binding domain (9), they have dramatically different effects on genome replication. The small form is required for replication,

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whereas the large form is a potent trans-dominant inhibitor (10, 11).

The last four amino acids of large delta antigen are Cys-Arg-Pro-Gln-COOH. This COOH-terminal configuration, termed a CXXX box (where C is cysteine and X is any amino acid), has been implicated as a substrate for prenyltransferases that add to the cysteine 15 (farnesyl) or 20 (geranylgeranyl) carbon moieties derived from mevalonic acid (12-14). The resulting hydrophobic modification may aid in membrane association of the derivatized protein, as suggested for p21 Ras (15, 16) and lamin B (12, 17). We have now demonstrated that large delta antigen is similarly modified.

Other virions also contain suitable target sequences for prenylation. These sequences are near the carboxy terminus of the viral protein targeted, and may be in the form of CXXX boxes, but the cysteine may also be closer to the C-terminus, including a position as the C-terminal amino acid, as is the case of the core antigen of hepatitis B virus (HBV) and the nef gene product of HIV-1.

To determine whether large delta antigen is a substrate for prenylation, we labeled three cell lines, SAG, LAG, and GP4F, with [<sup>3</sup>H]mevalonic acid. GP4F cells are a derivative of NIH 3T3 cells (18). SAG (19) and LAG (20) cells are derivatives of GP4F cells that stably express the small and large delta antigens, respectively.

Labeled cell lysates were analyzed on immunoblots (Fig. 1A) to detect steady-state amounts of small and large delta antigen. The lysates were also subjected to immunoprecipitation with an antibody to the delta antigens (anti-delta), SDS polyacrylamide gel electrophoresis (SDS-PAGE), and fluorography (Fig. 1B).

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In more detail, referring to Fig. 1, large delta antigen is shown to be prenylated in cultured cells. The cell lines SAG (19) (lane 1), LAG (20) (lane 2), and GP4F (18) (lane 3) were grown overnight in Lovastatin (25  $\mu$ M) and (R,S)-[5-<sup>3</sup>H]mevalonate (140 mM) (30), and lysed in RIPA buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS] (20). (A) Aliquots were subjected to immunoblot analysis (11). The blot was treated with serum from an HDV-infected patient that contained antibody to delta antigen ( $\alpha$ - $\delta$ Ag) and horseradish peroxidase-conjugated rabbit antibody to human immunoglobulin G (IgG) (Promega), followed by chemiluminescence (Amersham) development. (B) Immunoprecipitates (with  $\alpha$ - $\delta$ Ag) from cell extracts were subjected to SDS-PAGE and fluorography. As shown in Figure 1, S, small delta antigen, L, large delta antigen. Molecular size markers are shown at the left (in kilodaltons).

Thus, the large, but not the small, antigen was labeled with [<sup>3</sup>H]mevalonic acid, suggesting that large delta antigen undergoes prenylation in cultured cells.

We obtained similar results using *in vitro* translation reactions (13) performed in the presence of [<sup>3</sup>H]proline or [<sup>3</sup>H]mevalonate (Fig. 2). Fig. 2. also shows mutation of Cys<sup>211</sup> of large delta antigen to Ser and loss of prenylation. *In vitro* translation reactions were performed with rabbit reticulocyte lysates (Promega) in the presence of either (A) L-[2,3,4,5-<sup>3</sup>H]proline (19  $\mu$ M) (94 Ci/mmol, Amersham) or (B), [<sup>3</sup>H]mevalonate (200  $\mu$ M) (30). For (A) and (B), translation reactions contained small delta antigen mRNA (lane 1); large delta antigen mRNA (lane 2); water (lane 3); or large delta antigen (Cys<sup>211</sup>  $\rightarrow$  Ser) (20) mRNA (lane 4). A portion 20  $\mu$ l of each reaction was added to 1 ml of RIPA buffer,

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immunoprecipitated with  $\alpha$ - $\delta$ Ag, and analyzed as described (Fig. 1).

Both the small and the large antigens were labeled with [ $^3$ H]proline (Fig. 2A), whereas only the large isoform was labeled with [ $^3$ H]mevalonate (Fig. 2B). To determine whether modification by [ $^3$ H]mevalonate was dependent on the presence of Cys<sup>211</sup> in the terminal CXXX box, we constructed a mutant that contains a serine at this position (20). Cys<sup>211</sup> is the only cysteine in large delta antigen. Mutating Cys<sup>211</sup> to Ser did not interfere with the synthesis of large delta antigen (Fig. 2A) but abolished its modification by [ $^3$ H]mevalonate (Fig. 2B).

The specific type of mevalonate modification of large delta antigen appears to be geranylgeranyl rather than farnesyl (21). Although the first described CXXX boxes contained aliphatic residues at the first and second positions after Cys, other types of amino acids can be found in prenylation sites (13, 14). It is not clear whether the COOH-terminal sequence Cys-Arg-Pro-Gln-COOH, which differs from that of previously described CXXX boxes, implies the existence of a novel prenylation enzyme or whether it reflects a broader substrate specificity of known prenyltransferases.

For HDV particle formation, delta antigen and associated genomes are presumably targeted to cell membranes that contain HBV envelope proteins. We hypothesized that prenylation of large delta antigen could be involved in this process. We first examined whether large delta antigen was sufficient for HDV-like particle formation. HBV surface antigen (HBsAg) was expressed transiently in COS-7 cells together with small or large delta antigen. Virus-like particles consisting of delta antigen packaged into HBsAg-containing envelopes



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were analyzed by immunoprecipitation of clarified media supernatants with an antibody to HBsAg (anti-HBs).

Fig. 3 shows particle formation with large delta antigen and HBsAg parts. For panels (A) and (B),  
5 COS-7 cells were transiently transfected with the following plasmids: SV24H, which expresses HBV surface antigen (31), and SVLAg, which expresses small delta antigen (19) (lane 1); SV24H and SVL-large, which  
10 expresses large delta antigen (20) (lane 2); and calcium phosphate precipitate without DNA (lane 3). In (C) and (D), COS-7 cells were transfected with SV24H and SVL-large (lane 4); SV24H and SVL-large (Ser<sup>211</sup>) (20) (lane 5); and calcium phosphate precipitate without DNA (lane 6). For (A) and (C), 48 hours after transfection, HBsAg-  
15 containing particles were immunoprecipitated from 2-ml aliquots of clarified media supernatants with anti-HBs (31) and subjected to immunoblot (with  $\alpha$ - $\delta$ Ag) and chemiluminescence analyses as described (Fig. 1). For (B) and (D), the transfected cells were harvested in cell  
20 lysis buffer [50 mM Tris (pH 8.8), 2% SDS] with protease inhibitors (20), and aliquots subjected to protein immunoblot and chemiluminescence analyses. Molecular size markers are shown at the left (in kD).

The presence of delta antigen in the  
25 immunoprecipitates was assayed by immunoblot analysis (Fig. 3A). Although both small and large antigens were synthesized in the transfected cells (Fig. 3B), only the large isoform was incorporated into secreted HBsAg-containing particles (Fig. 3A). Similar selective  
30 packaging has been observed (22).

We then examined the function of mevalonate modification in this particle formation. One explanation for the preferred packaging of large delta antigen is

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that the small antigen lacks the CXXX box and therefore cannot undergo modification. The Cys<sup>211</sup> → Ser mutant of large delta antigen should behave like small delta antigen and not be packaged. This was indeed found to be the case. Whereas both wild-type and Ser<sup>211</sup> mutant large antigens were synthesized in transfected cells (Fig. 3D), only the wild-type form was packaged into particles (Fig. 3C). Thus, the mutated form of large delta antigen is not prenylated and cannot form particles with HBsAg.

Our results suggest that prenylation of large delta antigen is required for the formation and release of particles containing delta antigen and HBV surface antigens. The requirement of a prenylation site for productive viral infection is further suggested by other mutations of the CXXX box (23) and by the conservation of Cys<sup>211</sup> and a CXXX box motif among all sequenced HDV isolates (24).

The ability of large, but not small, delta antigen to be prenylated and packaged into virus particles further highlights the significance of the mutation-induced heterogeneity at the termination codon of the small delta antigen. During HDV replication, S genomes (encoding the small antigen) mutate to L genomes (encoding the large antigen). At least two effects attributable to this mutation can be distinguished (see Fig. 4). Fig. 4 shows the regulatory switch of S genomes to L genomes. During replication, S genomes encoding the small delta antigen mutate to L genomes, which encode the large delta antigen. This single base mutation has two effects on the COOH-terminus of delta antigen. The first is to change the nature of the COOH-terminal amino acid; Pro (P), which enhances genome replication (20), is replaced by Gln (Q), resulting in inhibition of genome replication. The second effect is the creation of a

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target prenylation site (CRPQ), C, cysteine; R, arginine; P, proline; Q, glutamine.

Thus, the first effect is the conversion of an enhancer of genome replication (small delta antigen) into a potent trans-dominant inhibitor (large delta antigen) (10, 11). This dramatic difference in function appears to be determined solely by the nature of the COOH-terminal amino acid with proline being sufficient to confer enhancer activity (11, 25). The second effect is the addition of a CXXX box to delta antigen, which allows the protein to be prenylated and presumably promotes its incorporation into HBsAg-containing particles. The combined effects of the switch from production of small to large delta antigen thus appear to have two roles: to suppress further genome replication and to promote the onset of packaging and virion morphogenesis.

Our results suggest prenylation as a new target for anti-HDV therapy and for antiviral therapy with respect to other viruses with prenylated proteins. Such therapy is directed at inhibiting virion morphogenesis, production, release and uncoating (functionally the reverse reaction of virion morphogenesis). In light of the increasingly apparent degeneracy of the four C-terminal amino acids required to function as a prenylation substrate, a cysteine located at any of these C-terminal positions is also considered to identify a potential target of antiprenylation therapy.

Several strategies designed to interfere with the prenylation stage of the HDV life cycle may be considered, including drugs that inhibit enzymes along the prenylation pathway, and CXXX box analogs. Both therapies have been considered for the inhibition of ras-mediated oncogenic transformation (26). Tetrapeptides that correspond to the CXXX box of p21 Ha-Ras inhibit

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prenylation of p21 Ha-Ras *in vitro* (27). Finally, the dual function of large delta antigen in the HDV life cycle suggests a further refinement of a proposed (11) defective interfering particle- (DIP) (28) like therapy aimed at cells infected with actively replicating S genomes. Because L genomes require a source of small delta antigen for replication (19, 29) but, once replicated, produce a potent trans-dominant inhibitor of further replication, a therapeutically administered L genome DIP could be specific for infected cells, as well as possess an inherent shut-off mechanism (11). If the L genome also contained the Cys<sup>211</sup> to Ser mutation, it could encode a delta antigen that not only inhibits replication but also affects packaging.

Accordingly, new approaches to antiviral therapy and inhibition of viral morphogenesis focus on inhibition of the prenylation of, or post-prenylation reactions of, at least one viral protein. This may be effected by contacting cells infected with the target virus with an effective amount of an agent which inhibits the prenylation of, or post-prenylation reactions of, at least one viral protein. Such agents include inhibitors of formation of the prenyl groups which are derivative of the mevalonate synthesis pathway. Other agents include decoys for the target sequence for prenylation, including small peptides, including tetrapeptides and other compounds which mimic the surroundings of the cysteine residue to be prenylated. For example, Reiss, Y., et al. Cell (1990) 62:81-88 report prenylation inhibition by C-A-A-X tetrapeptides. As set forth above, the cysteine residue to be prenylated is generally found at the carboxy terminus of the target protein; although the most common target sequence involves a CXXX box, cysteines positioned closer to the C-terminus may also be targeted;

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thus, the relevant peptides may include those of the form  
XCXX, XXCX, and XXXC. Other agents include derivatives  
and mimics of prenyl groups themselves. Other suitable  
agents include inhibitors of the prenyltransferase  
5 enzymes and of enzymes that catalyze post-prenylation  
reactions.

#### Assay of Candidate Inhibitors

The present invention also provides a method to  
10 screen candidate drugs as prenylation inhibitors by  
taking advantage of the requirement for prenylation in  
order to effect secretion of certain prenylated proteins.  
For those proteins for which secretion requires  
prenylation, the assay can be conducted in a direct and  
15 simple manner. Cells that secrete, or that have been  
modified to secrete, a first protein whose secretion is  
dependent on prenylation are used as the experimental  
cells. A second protein which does not depend on  
prenylation for secretion is used as a control. This  
20 control protein may be secreted by the same or different  
host cells as the first protein. The candidate drug is  
applied to cells that secrete both proteins, or to  
matched sets of cells that secrete each. Secretion can  
readily be assessed by assaying the cell supernatants for  
25 the presence or absence of the first and second secreted  
proteins using, for example, routine ELISA assays.  
Successful candidate drugs will not inhibit the secretion  
of the control protein, but will inhibit the secretion of  
the protein in the test sample wherein prenylation is  
30 required for secretion.

The large delta antigen of HDV is a viral  
protein for which prenylation is a prerequisite for  
secretion. Thus, this protein forms, itself a key part  
of a useful test system for the assay. Cells that are  
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modified to secrete a protein for which prenylation is not required can be used as controls. If large delta antigen is used as the test protein, it is advantageous to use HBsAg as the control protein in the same cell  
5 since HBsAg is also required for secretion of delta antigen.

The foregoing assay, of course, requires that the inhibitor interfere with the prenylation system for large delta antigen or for any other prenylation-  
10 controlled secreted protein used in the assay. A range of prenyl transferases and prenyl groups is known to apply to various proteins for which prenylation inhibitors are required or sought. Some of these proteins are not secreted, whether they are prenylated or  
15 not; one such example is the protein product of the ras oncogene.

Nevertheless, the assay system described can be employed to screen for inhibitors of prenylation in these nonsecreted proteins by providing the target "CXXX" box  
20 characteristic of the nonsecreted protein in place of the corresponding "CXXX" box of the secreted one. The resulting chimeric protein will exhibit the prenylation characteristics of the imported "CXXX" box characteristic of the nonsecreted protein, but retain the ability of the  
25 host secreted protein to be passed to the supernatant for assay. Thus, the range of target proteins for which prenylation inhibitors are sought by use of the assay can be expanded to nonsecreted proteins.

The presence of a control system which provides  
30 secreted protein not dependent on prenylation is critical. The presence of this control allows candidate inhibitors which merely are toxic to the cells, or which inhibit secretion in general, to be discarded. Prenylation inhibitors identified by one of the  
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variations of the above described assay are expected to find use not only in the inhibition of viruses, but also in other processes or disease states -- including but not limited to cancer -- in which a prenylated protein is found to be involved.

Evidently, prenylation of viral proteins is a prerequisite for additional post-prenylation reactions of the proteins such as proteolysis and carboxymethylation. The essential sequence of steps can be interfered with at the most convenient point for the viral protein in question.

#### Administration of the Inhibitors

Additional viral proteins subject to prenylation can be obtained by screening amino acid sequence data banks for viral proteins which contain a "CXXX" box at the C-terminus. An illustrative list of such proteins includes, for example, specific proteins of HAV, HCV, HSV, CMV, VZV, influenza virus, plant viruses such as tobacco mosaic satellite virus and barley stripe mosaic virus, core antigen of hepatitis B virus and the nef gene product of HIV I, as set forth above. These candidates for suitable prenylation targets can be validated in a manner similar to that described above by providing labeled mevalonic acid to cells infected with or containing the appropriate viruses or viral gene products, and assessing the prenylation status of the viral proteins obtained using incorporation of label as the criterion. Furthermore, the role of prenylation in the morphogenesis of the respective virions, and its suitability as a target for anti-viral therapy, can also be validated in a manner similar to that described above.

If viral morphogenesis, production, release or uncoating are to be inhibited in culture, suitable host

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cells are used to culture the virus, and the agents used in inhibiting prenylation or post prenylation reactions added to the medium. If the infected cells are contained in an animal subject, such as a mammalian subject or in particular a human or other primate subject, the agent used for the prenylation inhibition is generally introduced as a pharmaceutical formulation. Suitable formulations depending on the nature of the agent chosen may be found in Remington's Pharmaceutical Sciences, latest edition, Mack Publishing Co., Easton, PA. The routes of administration include standard such routes, including administration by injection, oral administration, and transmucosal and transdermal administration. The choice of formulation will depend on the route of administration as well as the agent chosen. Suitable mixtures of agents can also be used as active ingredients. For administration to plants, formulations which are capable of conducting the active ingredients into plant cells are used as carriers.

The following references are listed according to the number which refers to them in the body of the specification:

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-16-

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- 5 24. Of 14 independent viral isolates sequenced, 13 code for Cys-Arg-Pro-Gln-COOH and 1 codes for Cys-Thr-Pro-Gln-COOH as the four terminal amino acids of large delta antigen [Wang, K.-S., et al., Nature (1986) 323:508; Makino, S., et al., *ibid.* (1987) 329:343; Kuo, M.Y.P., et al., J. Virol. (1988) 62:1855; Saldanha, J.A. et al., J. Gen. Virol. (1990) 71:1603; Xia, Y.-P., et al., (1990) 178:331; Imazeki, F. et al., J. Virol. (1990) 64:5594; Chao, Y.-C., et al., Hepatology (1991) 13:345; Deny, P. et al., J. Gen. Virol. (1991) 72:735].
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Claims

1. A method to inhibit virion morphogenesis, .  
production, release or uncoating which method comprises  
5 contacting cells infected with a virus with an effective  
amount of an agent which inhibits the prenylation or the  
post-prenylation reactions of said at least one viral  
protein .

10 ~~2.~~ The method of claim 1 wherein said agent is  
an inhibitor of prenyl group synthesis or wherein said  
agent is an inhibitor of prenyl transferase, or wherein  
said agent mimics a prenyl group or wherein said agent  
mimics the prenylation locus of the viral protein.

15 ~~3.~~ The method of claim 1 wherein said viral  
protein contains a C-terminal amino acid sequence of the  
formula CXXX, XCXX, XXCX or XXXC wherein C is cysteine  
and each X is independently any amino acid.

20 ~~4.~~ The method of claim 2 wherein said agent  
mimics said CXXX, XCXX, XXCX or XXXC

25 5. The method of claim 1 wherein said agent  
interferes with a post-prenylation reaction.

30 6. The method of claim 1 wherein said virion  
is hepatitis D virus (HDV) and said viral protein is the  
large delta antigen of said HDV.

35 ~~7.~~ The method of claim 6 wherein said  
inhibition is effected by a transdominant inhibitor of  
replication modified to resist prenylation.

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8. The method of claim 1 wherein said virion is human immunodeficiency virus and said viral protein is the nef protein.

5 9. The method of claim 1 wherein said cell is contained in an animal or plant subject and said contacting comprises administering said agent to said subject.

10 10. A method to screen candidate drugs as prenylation inhibitors which method comprises contacting cells which secrete or which have been modified to secrete a first protein containing a "CXXX" box and a second control protein wherein secretion of said first  
15 protein is dependent on prenylation and secretion of said second control protein is not dependent on prenylation, with said candidate drug under conditions wherein said control second protein is secreted, and determining the presence, absence or amount of  
20 said first protein secreted from said cells, wherein a candidate drug which decreases or abolishes the amount of secreted first protein is said effective prenylation inhibitor.

25 11. The method of claim 10 wherein said first protein is a large delta antigen.

30 12. The method of claim 10 wherein said first protein is a chimera consisting of a natively secreted protein which has been modified to contain, in place of its "CXXX" box, the "CXXX" box of a different protein.

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Abstract

Viral morphogenesis, production, release or  
uncoating can be inhibited by effecting inhibition of  
5 prenylation of, or inhibition of post-prenylation  
reactions of, at least one viral protein. The use of  
inhibitors of prenylation, and post-prenylation  
reactions, for example, inhibitors of the mevalonate and  
prenyl group synthesis pathways, inhibitors of prenyl  
10 group transferases and mimics of the prenylation target  
. CXXX box are disclosed.

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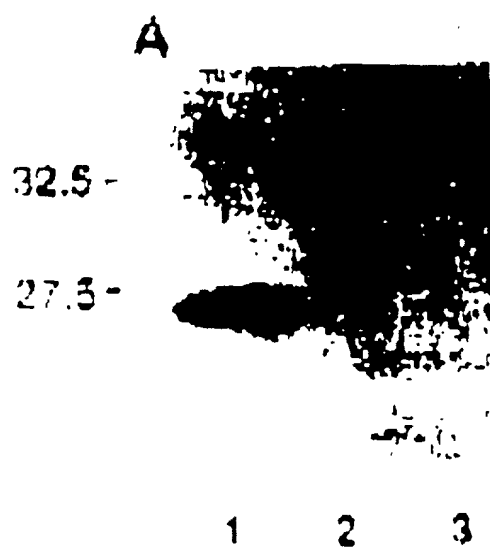


Fig. 1A

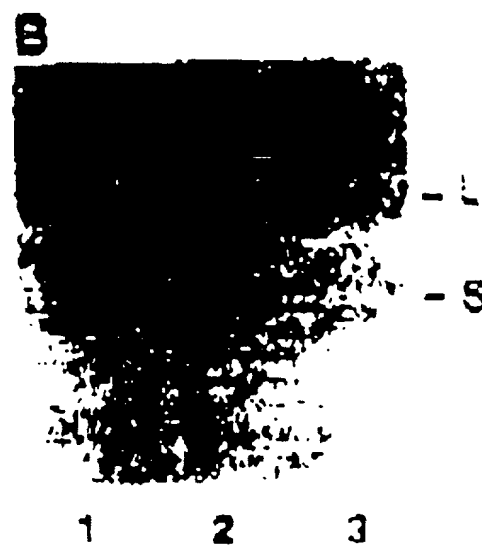


Fig. 1B



**A**

1 2 3

Fig. 3A

**B**

32.6 —  
27.5 —

1 2 3

Fig. 3B

**C**

4 5 6

Fig. 3C

**D**

4 5 6

Fig. 3D



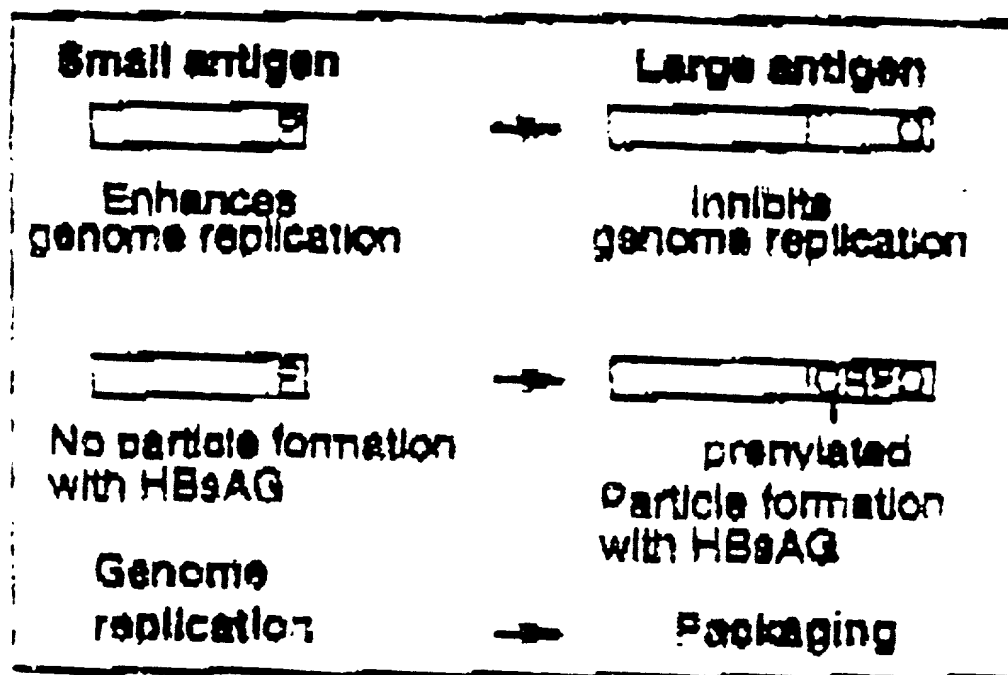


Fig. 4

## SEQUENCE LISTING

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<120> Method for Inhibition of Viral Morphogenesis

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<150> US 07/890,754

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## COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY

(Includes Reference to PCT International Applications)

ATTORNEY'S DOCKET NUMBER

4004-0524.20

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

METHOD FOR INHIBITION OF VIRAL MORPHOGENESIS

the specification of which (check only one item below):

☐ is attached hereto.

☒ was filed as United States application

Serial No. 08/347,448

on 29 November 1994

and was amended

on \_\_\_\_\_ (if applicable).

☒ was filed as PCT international application

Number PCT/US93/05247

on 1 June 1993

and was amended under PCT Article 19

on \_\_\_\_\_ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

## PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. 119:

COUNTRY (if PCT, indicate PCT)	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 USC 119
United States	07/890,754	29 May 1992	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO
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			<input type="checkbox"/> YES <input type="checkbox"/> NO

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

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U.S. APPLICATIONS		STATUS (Check one)		
U.S. APPLICATION NUMBER	U.S. FILING DATE	PATENTED	PENDING	ABANDONED

PCT APPLICATIONS DESIGNATING THE U.S.		
PCT APPLICATION NO.	PCT FILING DATE	U.S. SERIAL NUMBER ASSIGNED (if any)

**POWER OF ATTORNEY:** As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. (List name and registration number) William H. Bera - Reg No. 26,962, Karl Bascovic - Reg No. 29,907, Felissa H. Cagan - Reg No. 36,089, Thomas E. Clott - Reg No. 21,013, Alan E. Dow - Reg No. 36,123, Patricia M. Drost - Reg No. 29,780, Nancy Joyce Greasy - Reg No. 29,218, Bill Kennedy - Reg No. 33,407, Paul C. Kimball - Reg No. 34,841, Susan K. Lehnardt - Reg No. 33,843, Timothy J. Lichtigow - Reg No. 36,864, Shmuel Livnat - Reg No. 33,849, Barbara J. Luther - Reg No. 33,964, Gladys H. Mervoy - Reg No. 32,430, Kate H. Murashige - Reg No. 29,969, Jackie H. Nakamura - Reg No. 36,968, Freddie K. Park - Reg No. 36,836, Paul F. Schenck - Reg No. 27,263, Lynn E. Schwering - Reg No. P37,233, James R. Shay - Reg No. 32,082, Debra A. Shells - Reg No. 33,308, E. Thomas Whedcock - Reg No. 29,826

**Send Correspondence to:**

Kate H. Murashige  
Morrison & Foerster  
2000 Pennsylvania Avenue, Washington, DC 20006-1888

**Direct Telephone Calls to:**  
(name and telephone number)

Kate H. Murashige  
(202) 887-1533

201	FULL NAME OF INVENTOR	FAMILY NAME GLENN	FIRST GIVEN NAME JEFFREY	SECOND GIVEN NAME
	RESIDENCE & CITIZENSHIP	CITY Palo Alto	STATE OR FOREIGN COUNTRY California	COUNTRY OF CITIZENSHIP United States
	POST OFFICE ADDRESS	POST OFFICE ADDRESS 1130 Welch Road	CITY Palo Alto	STATE & ZIP CODE/COUNTRY California-94304
202	FULL NAME OF INVENTOR	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
	POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE & ZIP CODE/COUNTRY
203	FULL NAME OF INVENTOR	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
	POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE & ZIP CODE/COUNTRY

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SIGNATURE OF INVENTOR 201

DATE

12/15/94

SIGNATURE OF INVENTOR 202

DATE

SIGNATURE OF INVENTOR 203

DATE